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ydaG and *ydbA* of *Lactococcus lactis* Encode a Heterodimeric ATP-binding Cassette-type Multidrug Transporter*

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Multidrug resistance (MDR)-type transporters mediate the active extrusion of structurally and functionally dissimilar compounds from the cells, thereby rendering cells resistant to a range of drugs. The *ydaG* and *ydbA* genes of *Lactococcus lactis* encode two ATP-binding cassette half-transporters, which both share homology with MDR proteins such as LmrA from *L. lactis* or the mammalian P-glycoprotein. The *ydaG/ydbA* genes were cloned and expressed separately and jointly in *L. lactis* using the nisin-inducible system. When both proteins are co-expressed, several structurally dissimilar drugs such as ethidium, daunomycin, and BCECF-AM are extruded from the cell. YdaG and YdbA could be co-purified as a stable heterodimer. ATPase activity was found to be associated with the YdaG/YdbA heterodimer only and not with the individual subunits. Both the *ydaG* and *ydbA* genes are up-regulated in multidrug-resistant *L. lactis* strains selected for growth in the presence of a variety of toxic compounds. This is the first demonstration of a functional heterodimeric ATP-binding cassette-type MDR transporter.

Multidrug resistance is a phenomenon that has first been attributed to the active efflux of drugs that renders tumor cells resistant toward structurally unrelated compounds, thereby causing a serious problem in chemotherapy of cancer (1). Later, it was realized that similar transporters in bacteria are responsible for multiple resistance to antibiotics and other toxic compounds (2). The wide spread of (multi)drug-resistant microorganisms is a serious treat to the effectiveness of antibiotics, and accordingly, previously curable infectious diseases reemerge, posing an increasing public health problem (3–5).

The extrusion of noxious agents which are unrelated in terms of their chemical structures is catalyzed by so-called multidrug resistance (MDR)[1] transporters. These transporters can be divided into two major classes on the basis of bioenergetic and homology criteria: 1) ATP-binding cassette (ABC) primary active transporters, which utilize the free energy of ATP hydrolysis to drive substrate transport across the lipid bilayer; and 2) secondary transporters, which use the proton or sodium motive force for substrate transport. The latter class includes most of the bacterial multidrug efflux systems described to date (6–9). The former class comprises well characterized eukaryotic MDR transporters such as P-glycoprotein, MRP1, and BCRP (1, 10, 11). Surprisingly, only few homologs of eukaryotic ABC-type MDRs have been characterized in bacteria: LmrA from *Lactococcus lactis* (12), BmrA (YvcC) from *Bacillus subtilis* (13), and HorA of *Lactobacillus brevis* (14). These proteins are believed to function as homodimeric MDR transporters. Genomic sequences, however, show a ubiquitous distribution of putative MDR ABC-type pumps (15, 16), but these systems have not yet been linked to multidrug resistance in bacteria.

ABC transporters are usually composed of four domains: two hydrophobic transmembrane domains (TMDs) with typically six membrane-spanning α-helices and two membrane-associated, hydrophilic nucleotide binding domains (NBDs) (17–19). The TMDs are believed to accommodate the substrate binding site(s). The diversity of the substrates transported by members of ABC transporters is reflected by a low conservation of the TMDs sequences. In contrast, amino acid sequences of the NBDs are evolutionarily highly conserved with the Walker A and B motifs of the ATP binding domain as well as motifs unique to ABC transporters (*i.e.* the ABC signature, the histidine, and the glutamine loop) (19, 20). With many bacterial ABC transporters, the four domains are contained in separate proteins, whereas in eukaryotes, these domains are mostly fused to yield a so-called "full-transporter" that accommodates all domains in a single polypeptide (19, 21). In "half-transporters," one TMD is fused to an NBD, and these proteins either homo- or heterodimerize to form the active unit.

The Gram-positive bacterium *L. lactis* harbors at least two multidrug transporters (*i.e.* LmrP, a secondary drug extrusion system that mediates a drug/H^+ antiport activity, and LmrA, an ABC MDR transporter). In addition, some strains contain a plasmid-encoded LmrA homolog termed LmrB that is responsible for the extrusion of the bacteriocin, lactococcin (22). *L. lactis* exhibits several different MDR-like transport activities (23, 24) that are not explained by the presence of the well characterized transporters LmrA and LmrP. Most importantly, strains with a deletion of the *lmrP* (25) or *lmrA* gene (Fig. 2*B*) exhibit an unaltered ability to secrete ethidium from the cell. This implies that there must be other MDR-like transporters active in *L. lactis* that so far have escaped detection. Analysis of the recently published genome of *L. lactis* ssp. *lactis* IL1403 (26) indeed indicates the presence of another MDR-like transport system. Here we report on two open reading frames *ydaG*

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[1] The abbreviations used are: MDR, multidrug resistance; ABC, ATP-binding cassette; TMD, transmembrane domain; NBD, nucleotide binding domain; ST II, Streptag II; BCECF, 2′,7′-bis-(2-carboxyethyl)-5,6-carboxylfluorescein; AM, acetoxymethyl ester.

and *ydbA* that are predicted to be co-transcribed and that encode two separate MDR-like half-transporters. The data demonstrate that YdaG and YdbA heterodimerize to form a functional MDR transporter. This is the first report on a heterodimeric ABC MDR transporter.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*L. lactis* NZ9000 (Δ*lmrA*) which lacks the MDR transporter LmrA (22) (a kind gift from O. Gajic and J. Kok, Department of Genetics, University of Groningen) was used as a host for expression of pNG8048E-based plasmids with the nisin-controlled promoter (27). Multidrug-resistant mutants of *L. lactis* MG1363 selected for growth in the presence of ethidium (Eth^r), daunomycin (Dau^r), and rhodamine (Rho^r) were described by Bolhuis *et al.* (23). Cells were grown in M17 medium (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol when necessary.

Recombinant DNA Techniques—DNA manipulation was performed essentially as described by Sambrook *et al.* (28). The *ydaG* and *ydbA* genes were amplified by PCR from genomic DNA of *L. lactis* IL1403. NcoI and XbaI restriction sites flanking the genes were introduced to enable cloning into expression vector pNS8048E, resulting in pNSGA. This plasmid contains both *ydaG* and *ydbA* genes under control of the nisin-inducible promoter with a C-terminal Streptag II on YdbA. *ydaG* and *ydbA* were cloned separately using the same strategy, and this resulted in the plasmids pNSG and pNSA encoding for YdaG and YdbA with a C-terminal Streptag II, respectively. All cloned genes were verified by DNA sequencing to confirm that no PCR-borne mutations were introduced.

RNA Techniques—RT-PCR was used to assess the expression levels of *ydaG* and *ydbA* in a series of multidrug-resistant mutants of *L. lactis* MG1363 (23). Primer sets were chosen to amplify internal fragments of 320, 318, and 375 bp of *ydaG*, *ydbA*, and the *secY* gene, respectively (Table I). Total RNA was isolated from *L. lactis* strains using High Pure RNA isolation kit (Roche Applied Science), and RT-PCR reactions were performed with ReadyToGo RT-PCR beads according to the manufacturer's guidelines (Amersham Biosciences).

Preparation of Membrane Vesicles—Inside-out membrane vesicles were prepared from *L. lactis* NZ9000 (Δ*lmr*A) harboring different pNS8048E-based expression vectors. Control cells were transformed with pNG8048E, and these were subjected to the same treatment. Cells were grown at 30 °C up to an OD_{660} of 0.8, and the expression of the plasmid-encoded genes was induced by the addition of 0.2% (v/v) supernatant derived from an overnight culture of the nisin-producing strain *L. lactis* NZ9700 (~10 ng/ml Nisin A). Subsequently, cells were grown for 2 h, harvested by centrifugation, and washed once with 50 mM Tris-HCl, pH 7.0. Cells were resuspended in the same buffer and treated with lysozyme (10 mg/ml). After 30 min at 30 °C, 10 mM of $MgSO_4$ and 100 μg/ml of DNase were added, and the suspension was passed twice through a French pressure cell (15,000 p.s.i.). Cell debris was removed by two centrifugation steps at 13,000 × *g* (15 min at 4 °C), and the inside-out membrane vesicles were collected by ultracentrifugation at 125,000 × *g* for 1 h at 4 °C. The membranes were resuspended in 50 mM Tris-HCl, pH 7.0, supplemented with 10% glycerol and stored at −80 °C.

Strep-Tactin Affinity Purification—Inside-out membranes (~30 mg/ml total protein) containing overexpressed YdaG-ST II, YdbA-ST II, both YdaG and YdbA-ST II, or the control were solubilized in 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, and 1% (w/v) dodecylmaltoside. After 30 min on ice, insoluble material was removed by centrifugation for 20 min at 280,000 × *g* at 4 °C. Subsequently, the supernatant was subjected to one-step affinity purification on Strep-Tactin columns according to the manufacturer's instructions (IBA). Purified proteins were visualized by an SDS-polyacrylamide gel stained with SYPRO Ruby Protein Stain Gel (Molecular Probes, Inc., Eugene, OR) and Western blotting using Strep-Tactin alkaline phosphatase for detection (IBA).

ATPase Assay—The ATPase activity of purified YdbA and/or YdaG was determined using a colorimetric method of Lanzetta *at al* (29). Detergent-solubilized proteins were incubated at 30 °C in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NH_4Cl, 5 mM $MgSO_4$, 15 mM $MgCl_2$, 0.05% dodecylmaltoside, and 2.5 mM ATP. Samples of 30 μl were transferred to a 96-well microplate every 10 min, and the reaction was terminated by the addition of 150 μl of malachite green molybdate reagent. After 5 min, 34% citric acid was added to facilitate color development. Absorbance was measured after 50 min of incubation at room temperature at 600 nm and compared with a phosphorus standard.

Transport Assays—*L. lactis* cells induced for the various transporters were washed three times with 50 mM K-HEPES, pH 7.0, and 5 mM $MgSO_4$. For the daunomycin and BCECF-AM transport assays, the buffer was supplemented with 25 mM K_2SO_4. Cells were collected by centrifugation and resuspended to an OD_{660} of 0.5 in the buffer for the fluorescence measurements. For ethidium transport, cells were pre-energized for 5 min with 20 mM glucose, whereupon ethidium was added to final concentration of 10 μM. The fluorescence of the ethidium complex with polynucleotides was monitored at excitation and emission wavelengths of 500 and 580 nm, respectively (30). For BCECF-AM, pre-energized cells were incubated with the ionophores valinomycin and nigericin (1 μM each) to preclude any interference by the electrochemical pH gradient. BCECF-AM was added to a final concentration of 0.5 μM, and the fluorescence was monitored at excitation and emission wavelengths of 502 and 525 nm, respectively. For daunomycin transport measurements, 10 μM daunomycin was added to nonenergized *L. lactis* cells, and the passive influx of the compound into the cell was monitored by following the fluorescence quenching upon binding to polynucleotides. Fluorescence was monitored at excitation and emission wavelengths of 480 and 590 nm, respectively. Efflux was induced by the addition of 20 mM of glucose, whereupon the ionophores valinomycin and nigericin (1 μM each) were added to assess the contribution of the YdbA and YdaG proteins to the daunomycin efflux. Hoechst 33342 (1 μM) (Molecular Probes) transport assays were performed with glucose-energized cells. Fluorescence emission and excitation wavelengths were at 355 and 457 nm, respectively. All measurements were performed with a PerkinElmer Life Sciences model 50B fluorometer with magnetic stirred holder at 30 °C.

Other Techniques—Protein expression levels were determined by SDS-PAGE and Western blotting using polyvinylidene difluoride mem-

TABLE I
Sequence of primer sets used for RT-PCR

Primer name	Primer sequence	Expected product size
		bp
SecY1F	tacaactgctccagctacga	375
SecY2R	gttcctccaagagcgacaat	
YdaG1F	cgttcgtgaagcgacttaca	320
YdaG2R	tggcgttaatacggtccatc	
YdbA1F	ttgacagttgctgcttctcc	318
YdbA2R	aagaacataggccgttccac	

TABLE II
Comparison of amino acid sequence between different MDR systems

	Identity[a]					
	YdbA[b]	YdaG	LmrA	BmrA	C-Pgp	N-Pgp
	%	%	%	%	%	%
YdbA		21.3	22.5	24.4	25.6	25.7
YdaG	21.3		24.2	25.6	22.6	25.0
LmrA	22.5	24.2		42.1	27.1	27.6
BmrA	24.4	25.6	42.1		26.1	29.3
C-Pgp	25.6	22.6	27.1	26.1		38.7
N-Pgp	25.7	25.0	27.6	29.3	38.7	

[a] Percentage of identical residues in pairwise alignments using the ClustalW tool available on the World Wide Web at pbil.ibcp.fr/html/pbil_index.html.

[b] Shown are *L. lactis* YdaG (accession number AAK04408) YdbA (AAK04409), and LmrA (P97046); *B. subtilis* BmrA (D70031); and the N- and C-terminal halves (residues 1–640 and 641–1279, respectively) of the human multidrug resistance P-glycoprotein (NP 000918).

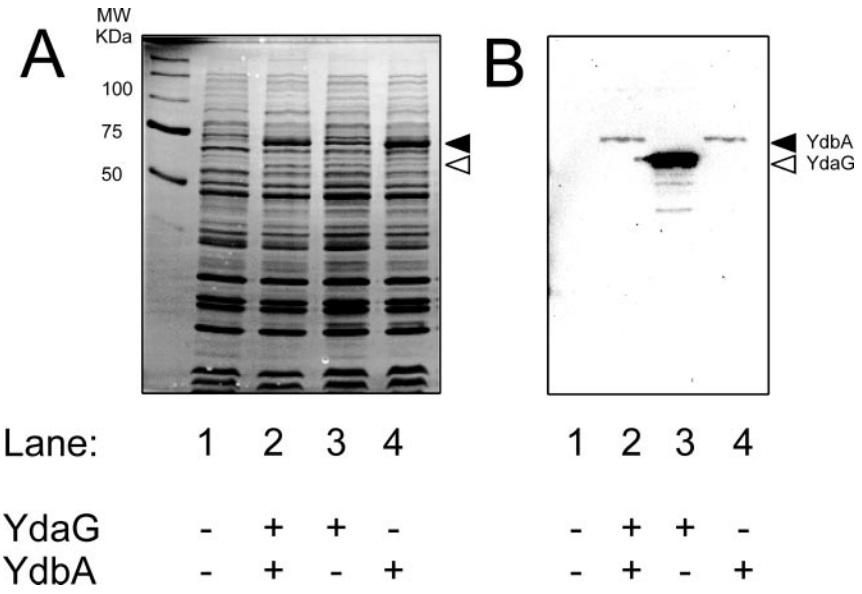

FIG. 1. **Overexpression of YdaG, YdbA, and YdaG/YdbA in *L. lactis* NZ9000 (Δ*lmrA*).** *A*, Coomassie Brilliant Blue-stained SDS-PAGE gel of inside-out membrane vesicles (20 μg of protein/lane) prepared from cells harboring the empty vector (*lane 1*) or vectors expressing YdaG/YdbA with an ST II on YdbA (*lane 2*), YdaG-ST II (*lane 3*), or YdbA-ST II (*lane 4*). *B*, Western blot of the SDS-PAGE gel of *A* stained with Strep-Tactin-alkaline phosphatase. The *arrows* indicate the positions of YdaG and YdbA. YdbA-ST II stained poorly with the Strep-Tactin-alkaline phosphatase, which is most likely due to partial truncation of the carboxyl-terminal tag.

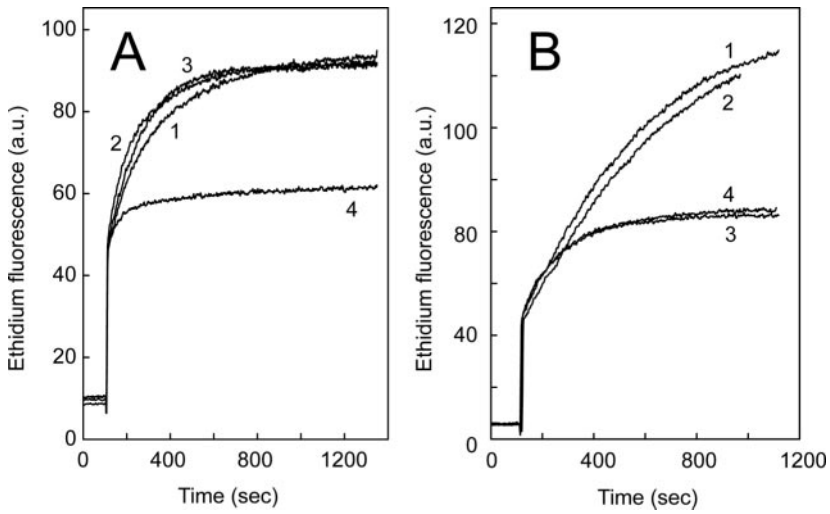

FIG. 2. **YdaG- and YdbA-mediated ethidium efflux from *L. lactis*.** *A*, accumulation of ethidium bromide by glucose-energized cells of *L. lactis* NZ9000 (Δ*lmr*A) harboring the control plasmid pNG8048E (*line 1*) and plasmids for expression of YdaG (*line 2*), YdbA (*line 3*), and YdaG/YdbA (*line 4*). *B*, accumulation of ethidium bromide in *L. lactis* NZ9000 (*lines 2* and *4*) and NZ9000 (Δ*lmr*A) (*lines 1* and *3*) in the absence (*lines 1* and *2*) and presence (*lines 3* and *4*) of glucose. Washed cell suspensions (0.1 mg of protein/ml) were pre-energized, where necessary, for 5 min with 20 mM glucose as indicated, whereupon ethidium bromide was added to a final concentration of 10 μM. Fluorescence of ethidium was monitored over time. *a.u.*, arbitrary units.

branes. Proteins was detected with Strep-Tactin alkaline phosphatase conjugates directed against Streptag II (IBA, Germany) and visualized by chemiluminescence with CDP-Star (Roche Applied Science) and imaged on a Lumni Imager (Roche Applied Science).

RESULTS

Identification and Overproduction of the ABC Half-transporters YdaG and YdbA in L. lactis—Genome sequencing analysis of *L. lactis* ssp. *lactis* IL1403 (26) revealed the presence of two adjacent open reading frames that encode ABC half-transporters. The respective gene products YdaG and YdbA show significant sequence homology to the *L. lactis* LmrA protein and other ABC multidrug transporters (Table II), suggesting a possible role in multidrug resistance. The *ydaG* and *ydbA* genes were amplified by PCR from the *L. lactis* IL1403 genome and cloned into the pNS8048E expression vector, which is a derivative of pNG8048E that allows gene expression under control of the tightly regulated, inducible *nisA* promoter (27). The pNS8048E vector was created by insertion of an XbaI and HindIII DNA fragment encoding the Streptag II (ST II) and stop codon into pNG8048E. This allows the in-frame fusion of the ST II to the 5′ termini of the cloned genes, thus yielding the following expression constructs: 1) pNSG containing *ydaG* with a ST II; 2) pNSA containing *ydbA* with a ST II; and 3) pNSGA containing both *ydaG* and *ydbA* with a ST II localized on YdbA. The expression vectors were transformed to *L. lactis* NZ9000 (Δ*lmrA*), a strain that lacks the *lmrA* gene and that was used to preclude any background drug extrusion activity of this transporter. Cells were grown in the presence of nisin to induce the expression of the various genes, and subsequently, membranes were isolated for protein expression analysis. Membranes derived from *L. lactis* NZ9000 (Δ*lmrA*) harboring pNSG, pNSA, or pNSGA showed on Coomassie Brilliant Blue-stained SDS-PAGE the presence of protein bands with the expected molecular sizes: YdaG (63 kDa), YdbA (74 kDa), and YdaG/YdbA, respectively (Fig. 1*A*). These polypeptide bands were absent in membranes derived from *L. lactis* NZ9000 (Δ*lmrA*) harboring the control vector pNG8048E. Western blotting with Strep-Tactin conjugated to alkaline phosphatase directed against the ST II (ST II is Streptag) confirmed the presence of the tagged YdaG and YdbA proteins (Fig. 1*B*).

YdaG and YdbA Mediate Ethidium Bromide Extrusion from L. lactis Cells—Ethidium bromide is a toxic compound that is widely used to detect the activity of multidrug resistance efflux pumps (25, 31, 32). Ethidium becomes fluorescent upon intercalation with DNA, and this property can be used conveniently to monitor its influx into the cell. The addition of ethidium to glucose-energized *L. lactis* NZ9000 cells resulted in a rapid increase of ethidium fluorescence (Fig. 2*B*). An identical influx pattern was observed with *L. lactis* NZ9000 (Δ*lmrA*) cells that lack the wild-type *lmrA* gene (Fig. 2*B*), indicating that LmrA

does not significantly contribute to ethidium efflux in these cells. When *L. lactis* NZ9000 (Δ*lmrA*) cells were used that expressed both YdaG and YdbA, a strongly reduced influx of ethidium was observed. In contrast, overexpression of YdaG or YdbA alone resulted in a pattern indistinguishable from the control plasmid and thus did not decrease the influx of ethidium (Fig. 2*A*). These observations indicate that both YdaG and YdbA are needed for active extrusion of ethidium, suggesting that these proteins interact to form a functional MDR transporter.

YdaG and YdbA Function as a Multidrug Transporter—In addition to ethidium, several other compounds were tested as possible substrates for the YdaG and YdbA transporters. The hydrophobic noncharged ester BCECF-AM is widely used for intracellular pH measurements. As an ester, BCECF readily diffuses across the cytoplasmic membrane, whereupon it is hydrolyzed by a nonspecific esterase that releases the membrane-impermeable fluorescent BCECF in the cytosol (33). Active efflux of BCECF-AM by the human multidrug transporter P-glycoprotein was demonstrated in NIH-3T3 mouse fibroblasts. In such assays, extrusion of BCECF-AM was assumed to occur from the membrane before the compound reached the cytoplasm, since the hydrolysis by esterases was found to be not rate-limiting for fluorescence development (34). Moreover, a multidrug-resistant mutant of *L. lactis* (Eth^r) that shows an increased resistance to ethidium bromide also rapidly expels the BCECF-AM in an ATP-dependent manner (24). A rapid development of BCECF fluorescence was observed when BCECF-AM was added to *L. lactis* NZ9000 (Δ*lmrA*) control cells or to cells that expressed YdaG or YdbA independently (Fig. 3). The fluorescence development was greatly reduced when cells were used that expressed YdaG and YdbA simultaneously (Fig. 3). The rate of BCECF-AM influx was not altered when cells were incubated with the ionophores nigericin and valinomycin, showing a similar behavior as ethidium-resistant (or Eth^r) *L. lactis* cells (24) and ruling out a proton motive force-dependent extrusion mechanism.

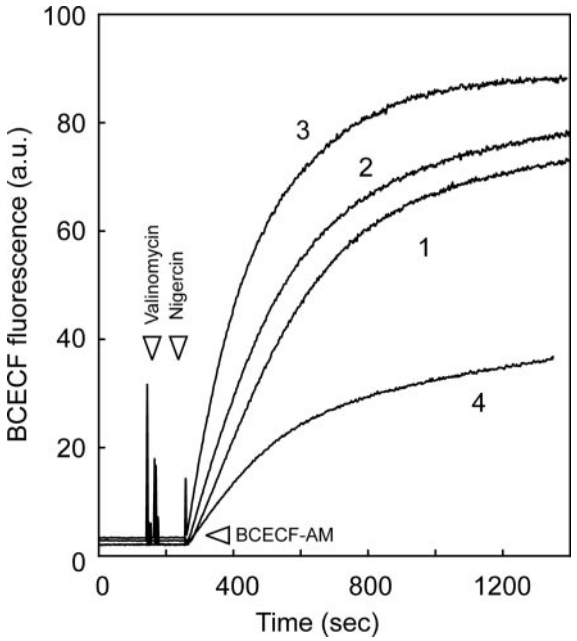

FIG. 3. **YdaG- and YdbA-mediated BCECF extrusion from *L. lactis* cells.** BCECF-AM influx was measured with *L. lactis* NZ9000 (Δ*lmr*A) cells harboring the control plasmid pNG8048E (*line 1*) and plasmids encoding YdaG (*line 2*), YdbA (*line 3*), and YdaG/YdbA (*line 4*). Washed cell suspensions (0.1 mg of protein/ml) were pre-energized for 5 min with 20 mM glucose in the presence of the ionophores valinomycin and nigericin (1 μM each). After the addition of BCECF-AM (0.5 μM), the BCECF fluorescence was followed in time. *a.u.*, arbitrary units.

The antitumor drug daunomycin is excreted by several human MDR transporters including P-glycoprotein, MRP1, and BCRP (for reviews, see Refs. 1 and 10). Furthermore, a high rate of daunomycin efflux was observed from an ethidium-resistant strain of *L. lactis* (23). The fluorescence of daunomycin is quenched upon binding to polynucleotides, and this feature can be used to monitor its influx. When daunomycin is added to *L. lactis* cells, a rapid fluorescence increase is observed due to the addition of drug followed by a drop in the fluorescence signal caused by entrance of the drug into the cell and binding to intracellular polynucleotides. Upon energization of the cells with glucose, all tested strains exhibited similar rates of daunomycin efflux (Fig. 4). However, upon the addition of the ionophores valinomycin and nigericin, which dissipate the proton motive force, only cells expressing both YdaG and YdbA were able to maintain a high fluorescence level

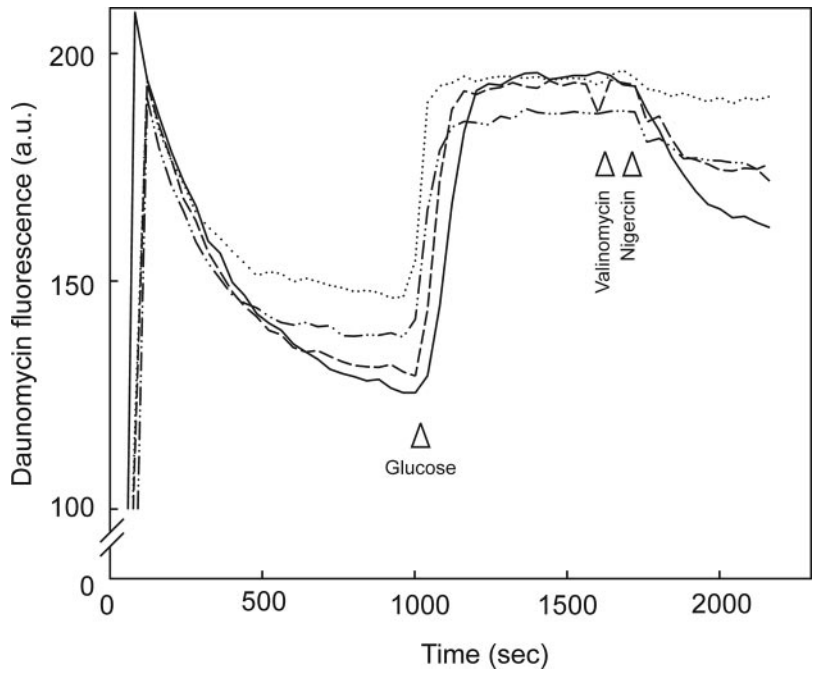

FIG. 4. **YdaG- and YdbA-mediated daunomycin transport in *L. lactis* cells.** Transport of daunomycin by *L. lactis* NZ9000 (Δ*lmr*A) cells harboring the control plasmid pNG8048E (*solid line*) and plasmids encoding YdaG (*dashed line*), YdbA (*dashed* and *dotted line*), and YdaG/YdbA (*dotted line*). To a washed cell suspension (0.2 mg/ml), 10 μM daunomycin was added and the influx of daunomycin was followed in time as a quenching of the fluorescence by the binding to DNA. As indicated by the *arrow*, cells were energized with glucose to initiate the extrusion of daunomycin. Valinomycin and nigericin were added to discriminate between the endogenous proton motive force-dependent efflux activity and the activity of YdaG/YdbA. *a.u.*, arbitrary units.

(Fig. 4). These data indicate that YdaG plus YdbA mediates efflux of daunomycin.

Another widely used substrate for MDR transporters is the lipophilic compound Hoechst 33342 (13, 30, 35–37). Hoechst 33342 is a membrane-permeable nuclear stain that becomes fluorescent upon intercalation in the lipophilic membrane environment and upon binding to DNA (37). The addition of Hoechst 33342 to glucose-energized cells of *L. lactis* NZ9000 (Δ*lmrA*) control cells results in a rapid increase in fluorescence, which is most likely due to binding of the drug to double-stranded DNA (Fig. 5). However, when cells are used that overexpress YdaG and YdbA, the fluorescence development of the drug is greatly reduced, suggesting that Hoechst 33342 is actively exported by the YdaG/YdbA transporter. Cells overexpressing YdbA show a fluorescence development that is similar to the control, whereas cells overexpressing YdaG reproducibly exhibited a slightly lower level of Hoechst 33342 accumulation (Fig. 5). Taken together, these data demonstrate that YdaG and YdbA constitute a functional MDR transporter in *L. lactis*.

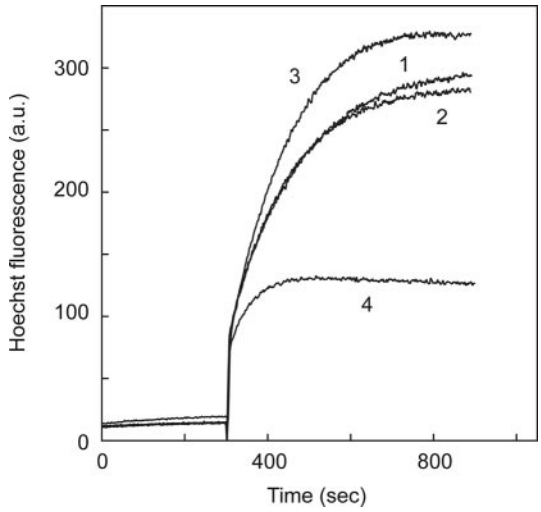

FIG. 5. **YdaG- and YdbA-mediated Hoechst 33342 extrusion from *L. lactis* cells.** Shown is transport of Hoechst 33342 by *L. lactis* NZ9000 (Δ*lmr*A) cells harboring the control plasmid (*line 1*) and the control plasmids for expression of YdaG (*line 2*), YdbA (*line 3*), and YdaG/YdbA (*line 4*). Cells were pre-energized for 5 min with glucose, whereupon 1 μM Hoechst 33342 was added. The fluorescence of Hoechst 33342 was followed in time. The cells expressing YdaG reproducibly (four independent experiments) exhibited a slightly elevated extrusion activity relative to control cells or cells expressing YdbA. *a.u.*, arbitrary units.

YdaG and YdbA Co-purify as a Heterodimer—Since YdaG and YdbA are both needed for the extrusion of various fluorescent compounds, it is likely that they form a heterodimer as a functional unit. To investigate whether YdaG and YdbA form stable dimers, a co-purification method was used. For this purpose, membranes were isolated from cells harboring the control vector or vectors that encode for YdaG and YdbA either independently or together. These constructs contained an ST II either on YdbA or on YdaG, whereas the co-expressed YdaG and YdbA harbored a ST II tag on YdbA only. Membranes were solubilized with dodecylmaltoside and subjected to one-step ST II affinity chromatography. Samples were analyzed by SDS-PAGE and Western blotting (Fig. 6, *A* and *B*). YdaG and YdbA migrate on SDS-PAGE as proteins with molecular masses of 63 and 74 kDa, respectively (Fig. 6*A*, *lanes 2* and *4*). When YdbA was purified from membranes containing both YdaG and YdbA-ST II, two polypeptide bands were obtained with molecular masses that corresponded to YdaG and YdbA, respectively (Fig. 6*A*, *lane 3*). The identity of these polypeptides was confirmed by Western blotting (Fig. 6*B*). From the relative intensities of YdbA and YdaG in the SYPRO Ruby-stained gel, it appears that both proteins are present in stoichiometric amounts, indicating the presence of a stable heterodimer.

The YdaG/YdbA Heterodimer Displays ATPase Activity—Our data show that YdaG and YdbA interact with each other to form a complex that can be purified, whereas both proteins are required to transport a range of unrelated drugs. To further explore whether both half-transporters are needed to form a functional unit, ATPase activity measurements were performed with the purified proteins. Only the co-purified complex of YdbA and YdaG showed a high ATPase activity, whereas the individual YdbA or YdaG proteins were essentially inactive (Fig. 7). These data further strengthen the notion that both proteins are required to form an active transporter.

ydaG and ydbA Are Up-regulated in Multidrug-resistant Strains of L. lactis—Previously, multidrug-resistant strains of

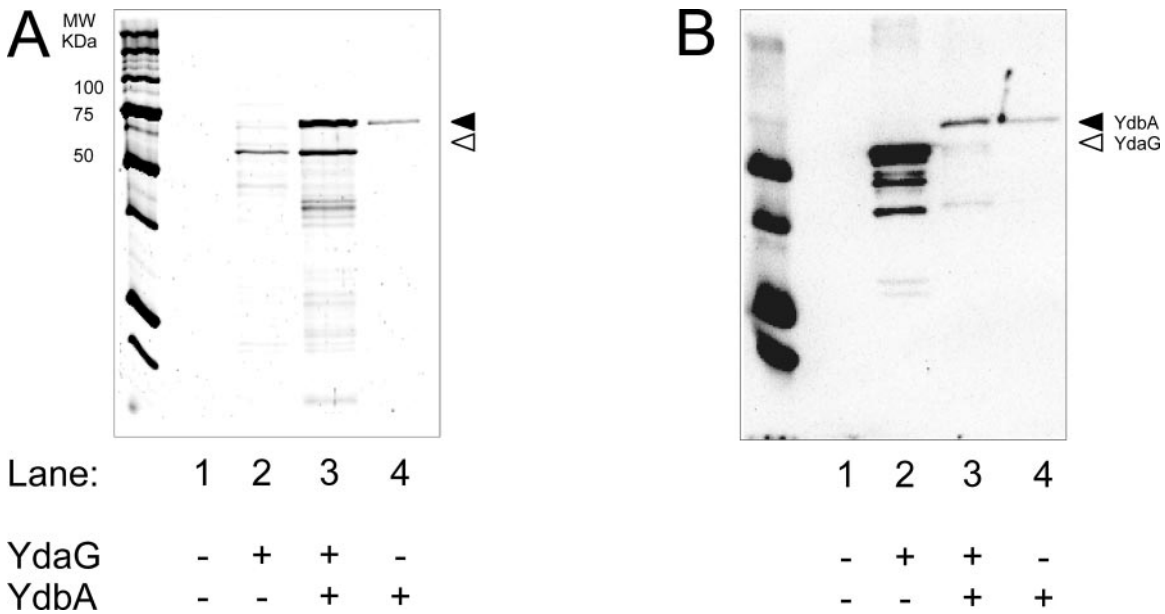

FIG. 6. **YdaG and YdbA co-purify as a heterodimer.** *A*, SYPRO Ruby-stained SDS-PAGE of Streptag II affinity-purified protein from membranes derived from control cells (*lane 1*) or from cells expressing YdaG-ST II (*lane 2*), YdaG and YdbA-ST II (*lane 3*), or YdbA-ST II (*lane 4*). *B*, Western blot of SDS-PAGE gel of *A* stained with Strep-Tactin alkaline phosphatase. The *arrows* indicate the positions of YdaG and YdbA.

L. lactis ssp. *lactis* MG1363 were selected for growth in the presence of high concentrations of ethidium bromide, daunomycin, and rhodamine 6G. The resulting mutants were found to be cross-resistant to a wide variety of structurally unrelated toxic compounds including the various selective drugs and quinine (23). To test whether *ydaG* and *ydbA* are involved in the multidrug resistance phenotype in *L. lactis*, we investigated their mRNA levels in the wild type and multidrug-resistant strains using an RT-PCR method. Both transporter genes are significantly up-regulated in all multidrug-resistant strains (Fig. 8). The expression of the *sec*Y gene was used as a control (Fig. 8). These results further support the hypothesis that the half-transporters YdbA and YdaG constitute a MDR heterodimeric transporter.

DISCUSSION

When *L. lactis* cells are challenged with increasing amounts of toxic compounds, mutants are obtained that are cross-resistant to a wide spectrum of structurally diverse drugs, many of which are typical substrates of the mammalian P-glycoprotein. This resistance is to a large extent due to the active secretion of these compounds from the cells. Previous biochemical and bioenergetic studies revealed that *L. lactis* contains at least two MDR transporters (*i.e.* the secondary transporter LmrP and the ABC transporter LmrA). The multidrug resistance phenotype of *L. lactis* mutants involves both proton motive force and ATP-dependent transport systems (23), but the exact identities of the systems responsible have remained obscure. Both LmrA and LmrP have been implicated in ethidium efflux in *L. lactis*, but a single deletion of *lmrP* (25) or *lmrA* (Fig. 2*B*) gene does not alter the ability of cells to extrude ethidium. So far, the presence of additional MDR-like transporters in *L. lactis* has not been explored. This prompted us to analyze the *L. lactis* IL1403 (26) genome for putative MDR pumps whose activity may contribute to the multidrug resistance phenotype in this organism. On the basis of homology with described MDR pumps such as human P-glycoprotein, LmrA of *L. lactis*, and BmrA of *B. subtilis*, we identified two new putative MDR transporters-encoding genes named *ydaG* and *ydbA*. These two genes are located on the chromosome next to another and are transcribed in the same direction. These genes have been predicted to form one co-transcribed unit (operon prediction available on the World Wide Web at www.tigr.org), and a similar genetic organization was observed in *L. lactis* strain MG1363.[2] The hydropathy profiles of the amino acid sequences and homology search suggest that both encode so called half-transporters possessing six putative membrane-spanning segments followed by cytoplasmically localized NBD domain.

To determine the function of YdaG and YdbA, the proteins were overproduced in *L. lactis* independently or together, using the nisin-inducible system. Next, the transport of a series of known fluorescent MDR substrates was tested. Strikingly, *L. lactis* cells overexpressing both YdaG and YdbA showed dramatically reduced levels of ethidium accumulation, which was not observed when the proteins were expressed independently. Similar results were obtained with the fluorescent dyes BCECF-AM, daunomycin, and Hoechst 33342. With the last substrate, a reproducible low activity was observed when YdaG was expressed independently of YdbA. Transport of these drugs seems to occur independently of the proton motive force, since the presence of the ionophores valinomycin and nigericin, which dissipate the $\Delta\psi$ and ΔpH, respectively, affected transport only marginally. This is consistent with the presumed ATP dependence of the transport function, which can be predicted on the basis of the presence of a NBD in both proteins. Our data

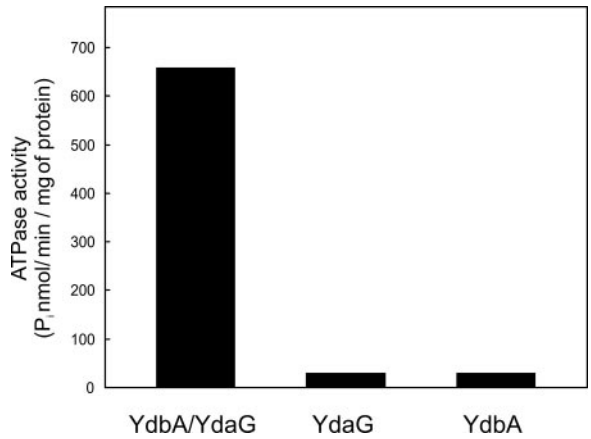

FIG. 7. **ATPase activity of purified YdaG/YdbA, YdaG, and YdbA.** Basal ATPase activity was measured as the release of P_i in time by an elution fraction containing YdaG and YdbA complex, YdaG, or YdbA as described under "Experimental Procedures."

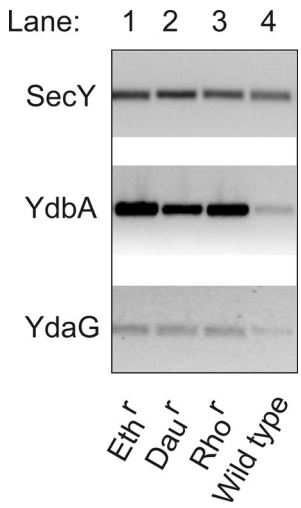

FIG. 8. **Expression of *ydbA* and *ydaG* in *L. lactis* MG1363 and derived multidrug resistance strains.** The expression of *ydbA*, *ydaG*, and the control *secY* gene was measured by RT-PCR using specific primer pairs and total RNA isolated from the parental *L. lactis* MG1363 (*lane 4*) and multidrug resistance derivatives obtained after selection by growth in the presence of ethidium (*lane 1*), daunomycin (*lane 2*), and rhodamine (*lane 3*).

suggest that YdaG and YdbA need to dimerize to form a functional ABC transporter. Evidence for heterodimerization was obtained by the observation that the proteins co-purify in a stoichiometric manner when subjected to affinity chromatography with only one of the proteins tagged. Moreover, the co-purified heterodimeric complex effectively hydrolyzes ATP, whereas separately purified YdbA or YdaG proteins exhibit only a minor ATPase activity. The later activity may arise from homodimer formation or alternatively from a minor fraction of heterodimer formation with the endogenous levels of YdaG or YdbA, respectively. Substrate such as ethidium and Hoechst 33342 did not stimulate the ATPase activity of the YdaG/YdbA heterodimer.[3] Lack of stimulation may be due to the already activated state of the transporter, or alternatively, the detergent-solubilized state might support this activated state. Similar observations have been reported for P-glycoprotein, where a low level of substrate activation of its ATPase activity was observed only for the membrane-reconstituted form of P-glycoprotein and not for the detergent-solubilized state (38). These

[2] J. Lubelski and R. van Merkerk, unpublished data.

[3] J. Lubelski, unpublished results.

questions will be addressed in future reconstitution studies.

In the cell, the multidrug resistance phenotype is often the result of several mechanisms, which may include various transporter systems. In particular, when cells are challenged with an increasing concentration of drugs, the emerging MDR phenotype may be a consequence of the elevated expression of various MDR transporters and/or mutations that affect the drug recognition spectrum. Strikingly, studies with wild type strains that contain either a deletion of the *lmrA* (this study) or *lmrP* gene (25) showed that the ethidium resistance and extrusion capacity of such cells remains unaltered questioning the importance of these two genes in the endogenous ethidium resistance of these cells. The lack of a drug-sensitive phenotype has been attributed to the elevated expression of other unknown drug transporters, but the identity of these systems has remained obscure. LmrP has been shown to effectively excrete ethidium (39), but since this protein is expressed only in the late logarithmic growth phase[4] it may not contribute to the drug resistance profile of the cells at the initiation of growth. Moreover, in contrast to previously published reports (30, 40–42), we do not observe an increased ethidium efflux rate from *L. lactis* NZ9000 wild type or Δ*lmrA* cells that overexpress the LmrA protein.[5] In a parallel experiment, overexpression of both YdaG and YdbA in *L. lactis* NZ9000 results in a marked ethidium extrusion (see also Fig. 2), which clearly marks this system as an ethidium extrusion system. We also noted an elevated expression of both *ydaG* and *ydbA* in several unrelated drug-resistant strains, among which is the ethidium-resistant *L. lactis* strain. The drug extrusion activities as observed in the ethidium-resistant strain were previously found to be insensitive to ionophores (23), which further suggests a role of YdaG and YdbA in the drug resistance. We cannot exclude the possibility that other drug transporters contribute to the drug resistance of these selected strains. However, both the functional assays and expression studies suggest that YdaG/YdbA contribute to the drug resistance patterns of the selected resistant *L. lactis* strains.

The active form of an ABC transporter usually constitutes four domains (*i.e.* two TMDs and two NBDs) (43). These proteins can exist either as a single polypeptide, as separate domains, or as fused domains in various organizational units. ABC half-transporters consist of a TMD fused with NBD, and they are believed to homo- or heterodimerize to form a full ABC transporter (44). Homodimerization has been experimentally demonstrated for several half-transporters, such as LmrA (40) and MsbA of *Escherichia coli* (45). There are several examples of eukaryotic ABC transporters that heterodimerize to form a functional unit such as, for instance, TAP1/TAP2, an ABC transporter associated with antigen presentation (46), and the *Drosophila* white, brown, and scarlet genes responsible for transport of pigment precursor to the eye (47). In the latter case, the substrate specificity depends on the heterodimerizing partner brown, scarlet, or white. Half-transporters localized in peroxisomes that belong to the ABCD subfamily, namely adrenoleukodystrophy protein, adrenoleukodystrophy-related protein, and peroxisomal protein of 70 kDa, were shown to homo- and heterodimerize as suggested by yeast two-hybrid studies. Accordingly, it was proposed that different combinations of such dimers vary in the substrate spectrum (48). Our data suggest that YdaG and YdbA heterodimerize to form an active MDR transporter. We therefore propose to rename YdaG and YdbA as the lactococcal multidrug resistance protein C (LmrC) and D (LmrD), respectively. Future studies will address the question whether LmrC and LmrD can also form homodimers and, if so, to what extent their substrate specificities differ from the LmrCD heterodimer.

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[4] P. Mazurkiewicz, unpublished data.

[5] J. Lubelski, B. van de Berg, G. Poelarends, R. Coole, C. Marbuef, P. Mazurkiewicz, W. N. Konings, A. J. M. Driessen, unpublished data.

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